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Gene transfer vector system derived from Coxsackie viruses

The present invention relates to a gene transfer vector system derived from Coxsackie Viruses, in particular Coxsackie Viruses of group B (CVB hereinbelow), especially for cardiac muscle-specific gene transfer.

Within the scope of the present invention, gene transfer means introducing DNA or RNA, generally denoted "foreign gene" here, into target cells in which the DNA and/or RNA provide additional functions and/or complement and/or compensate defective functions of the target cell, without the foreign gene being integrated into the genome of the target cell.

The present invention is particularly focused on the diagnosis, treatment and prevention of cardiac disorders which gain more and more importance in particular in the industrialized nations. After various genes for cardiac disorders have already been identified, and since it is generally expected that a multiplicity of pathological genes which can cause cardiac disorders are still going to be identified in the coming years, development of cardiac muscle-specific gene transfer systems for selective modulation of the endogenous gene activity of cardiac myocytes is of great clinical importance for future treatment of a multiplicity of congenital and acquired cardiac muscle disorders. However, ideal vector systems for controlled modulation of the endogenous gene activities of cardiac myocytes have not been available up until now.

Although various techniques for transfecting target cells of the cardiovascular system have been described previously, all of said techniques have specific disadvantages. In preclinical studies for somatic gene therapy of cardiovascular disorders, up until now especially replication-defective recombinant adenoviruses have been used, by which an adequate transfection efficiency is achieved only in vitro (Barr et al., Gene Therapy 1, 1994, 51). In contrast to recombinant adenoviruses, application of retroviral constructs (Nabel EG et al., Science 249, 1990, 1285) and of lipid/DNA/complexes (Nabel EG et al., Science 244, 1989, 1342) is limited by the low transfection efficiency of the gene transfer. Despite the advantages of adenovirus-mediated gene transfer with respect to high gene expression, the disadvantages of said method are the complementation of replication-inactive constructs, known from the literature,

the multiorgan tropism of adenoviruses and also the possible induction of a T-cell mediated immune response.

The known solutions, however, have quite a number of disadvantages in particular for gene transfer in the heart.

Thus, although adenoviral vectors have indisputable advantages, they are linked to the fundamental problem of virions passing too slowly through the endothelial barrier during infusion or coronary perfusion and further have the known problem of immunogenicity. For these reasons, adenoviral vectors are not optimal for gene transfer into the heart.

Also for retroviral vectors the rate of gene transfer into the heart and thus the efficiency are, similar to adenoviruses, most likely insufficient, since the heart is not a natural target organ of an infection of this type. However, the possibility of an insertion mutagenesis, which is connected with the risk of generating diseases such as, for example, cancer, must be regarded as the greatest disadvantage of retroviruses as vectors.

In contrast, the adeno-associated virus (AAV) can be used as a vector for cardiac muscle-specific gene therapy, since it is able to infect also quiescent cells. The disadvantage of AAV vectors, however, is the low packaging density of not more than approx. 4.7 kB so that important foreign genes are excluded and combinations of several foreign genes are impossible, respectively. A possible integration which, in contrast to the situation in vitro, happens randomly in vivo and can thus cause pathological effects must be regarded as another disadvantage.

The vector systems described so far are neither specific for cardiac muscle nor do they allow a therapeutically adequate efficiency. Moreover, none of said vector systems allows cytoplasmic replication of the vector backbone. All vector systems share the fact that the foreign gene to be transferred is expressed from a DNA so that there is a risk of stable integration into the genome of the target cell and mutagenesis.

As alternative, Felgner et al., PNAS, volume 84, pages 7413-7417, 1987 describe a transfection, called lipofection, of DNA into cell culture cells, wherein a liposome formulation is used in order to introduce recombinant DNA into cells and to express it there. Since lipofection is superior to viral vector systems with respect to safety, it is increasingly attempted to use this technique also for gene therapy of metabolic or tumor diseases. However, in most applications the efficiency is low, and especially in primary cultures or in applications in vivo, the known liposome systems are not very well suited so far.

Another approach is described by Kern et al. in "Coxsackievirus-verstärkter endosomolytischer Gentransfer in kontraktile Kardiomyozyten" [Coxsackie Virus-enhanced endosomolytic gene transfer in contractile cardiac myocytes], Verh. Dtsch. Ges. Path. Volume 81, page 611, 1997. Starting from the finding that the Coxsackie Virus has a tropism toward the heart, which is not understood so far, for lipofection they use for gene transfer into cardiac myocytes CVB3 particles which had been rendered replication-incompetent with UV radiation. CVB3 is a picornavirus having a single-stranded RNA genome of positive polarity and a genome size of only 7.4 kb. By comparison, adenoviruses have genomes of 48 kb in size.

Kandolf and Hofschneider, PNAS, volume 82, pages 4818/4822, 1985, describe a CVB3 variant with distinct tropism toward the heart. The complete nucleotide sequence of the cDNA of this infectious CVB3 variant is described in Klump et al., Journal of Virology, 1990, pages 1573-1783. The authors describe that the cDNA-derived virus has the same tropism and the same plaque morphology as the wild-type.

Another but not cardiospecific approach was described for the polio virus type 1 strain Sabin. The complete capsid region (P1) of the polio virus was replaced with a foreign gene, here an HIV gene. Transfection of the recombinant polio virus genome into cells and simultaneous infection of said cells with a recombinant vaccinia virus which provides the deleted P1 region in trans led to the generation of recombinant infectious polio virus particles. Infection merely with the recombinant polio virus genome led to expression of the foreign gene; Porter, J. Virol. 69, 1995, 1548 and Porter et al., J. Virol. 70, 1996, 2643.

Apart from the fact that it is not cardiospecific, the vector system described by Porter has, like AAV, the disadvantage that only short sequences, here 1.5 kB, can be used as foreign genes. Furthermore, Porter et al. describe the fact that polio viruses cause great changes in the physiology of the infected cells and that parenterally administered recombinant genomes are immunogenic.

Against this background, a polio virus-based vector system is not only unsuitable for cardiospecific gene transfer but has also generally disadvantages and risks which make the approach

described by Porter et al. not look very promising and successful.

In view of the above, it is an object of the present invention to provide a vector system which avoids the abovementioned disadvantages and enables in particular an effective, immunologically safe gene transfer into target cells, in particular into cardiac myocytes, which is, as possible, free of undesired side effects.

According to the invention, this object is achieved by a recombinant RNA molecule which can be translated at least partly in a target cell, comprising a noninfectious virus genome of Coxsackie Virus group B, preferably serotype B3 (CVB3 hereinbelow), and at least one foreign gene which causes a desired function in the target cell, for example within the framework of a gene therapy, whereby the RNA molecule is preferably replication-competent in the target cell.

The object underlying the invention is completely achieved in this manner.

In fact, the inventors of the present application have found that a recombinant RNA molecule based on the virus genome of CVB, preferably CVB3, or comparable serotypes makes it possible to construct an effective vector system, in particular for gene transfer into cardiac myocytes.

Within the scope of the present application, "noninfectious" means that the virus genome on its own is not capable of carrying out a complete infection cycle, because, for example, gene

sequences are missing or have been mutated such that they cannot fulfill their function of forming infectious virus particles.

"Translatable" here means that the RNA molecule, when introduced into the target cell by infection, is, at least partly, translated immediately into an amino acid sequence, whereby particular sequences of the RNA molecule ensure translation initiation.

The advantage of using the CVB or CVB3 genome, respectively, is the tropism of said virus toward the heart and the fact that during the CVB life cycle no DNA is formed, replication is carried out via an RNA-dependent RNA polymerase which the virus genome itself encodes. Thus there is no risk of integrating the transfected foreign gene into the genome of the target cell.

A higher translation efficiency is achieved, if the RNA genome is replication-competent, because in this case the RNA molecules are replicated in the cytoplasm so that, even after transfection or infection with only a few recombinant RNA molecules, a large number of such so-called replicons becomes available with time so that an adequate amount of foreign gene can be translated. In this way, a very efficient vector system is provided, which makes it possible to express foreign genes in target cells in a simple and secure manner, without any fear of the foreign genes integrating into the genome of the target cell.

It is in this connection particularly preferred, if in the virus genome parts of its coding sequence have been replaced by the at least one foreign gene.

This is an efficient and secure way of preventing that CVB goes through a complete infection cycle, since complementing the missing sequences in the target cell is impossible. The replacement of coding sequences by one or more foreign genes also makes it possible to transduce even large foreign genes.

In this connection, preference is given to replacing in the virus genome the sequences of its capsid proteins (VP1-VP4) and/or its protease 2A and/or 3C and/or its helicase 2C and/or its protein 2B, whereby additionally/alternatively the sequences of protease 2A and/or 3C are modified such that there is no cytotoxicity for the target cell.

Replacing one or more of the abovementioned sequences makes it possible to transduce relatively long foreign-gene sequences, while maintaining the original size of the virus genome. Maintaining the original length of the virus genome and also the ability to replicate makes it possible, due to the specificity for cardiac myocytes, that the recombinant RNA molecule is not only efficiently replicated but also translated with large yields in the target cell so that, even after infection with a few RNA molecules, efficient expression of the foreign gene in the target cells becomes possible. Reducing the virus genome portion essentially to the polymerase-encoding sequence 3D and the virus protein Vpg-encoding sequence 3B further ensures a very low risk of cytotoxicity. Even for vectors which addition-

ally also contain viral protease 3C it is guaranteed that there is no cytotoxicity.

In view of the above, the invention also relates to the use of an RNA molecule of this kind for generating a vector for gene therapy.

The CVB3 vector may be transfected into the target cells, for example, by means of the lipofection mentioned at the outset. However, preference is given to transduction by infection by means of a virion. A third possibility is to transfer the CVB3 vector via another viral vector, for example adenovirus.

In view of the above, the invention further relates to a recombinant, infectious virion derived from CVB, preferably CVB3, which contains the RNA molecule of the invention as genome.

In connection with this measure, it is advantageous that the CVB tropism toward the heart is used not only for replication and translation but also even for infection. It is known that CVB can be detected in cardiac muscle of mice already two days after oral or parenteral infection.

The fact that the RNA molecule, due to the virus genome portion present in the molecule, can be packaged readily into CVB capsid proteins, thus providing a safely infecting vector system which can be prepared easily, must be regarded as another advantage.

In this connection, it is preferred, if the virion corresponds in its structural proteins to a CVB, preferably CVB3.

This has the already mentioned advantage of utilizing the CVB tropism toward particular target cells, in particular the heart, both in infection with and packaging of the RNA molecule.

In view of the above, the invention further relates to a method for transducing a foreign gene into a target cell, comprising the steps:

- providing an RNA molecule or a virion of the abovementioned type, and

- infecting the target cell with the virion or transferring the RNA molecule by transfection.

The invention further relates to providing the RNA molecules according to the invention, which may be generated, for example, in stably transfected host cells.

In view of the above, the invention relates to a vector plasmid having at least one DNA sequence which codes for the RNA molecule according to the invention and having a promoter located in front of the DNA sequence.

In this way it is possible to produce the novel RNA molecule in large amounts and then either to transfer it immediately into the target cells or first to package it in the novel virion and then transduce it into the target cell via said virion.

Alternatively, however, it is also possible to introduce into the target cell a DNA construct which persists in the nucleus

there and generates the novel RNA molecule which then either replicates itself in the cytoplasm or is merely translated. Said DNA construct may also be provided by a different viral vector.

In view of the above, the invention further relates to a DNA construct which codes for an RNA molecule according to the invention and persists and transcribes but preferably does not replicate in a target cell, and to a recombinant virus, preferably adeno- or retrovirus, which codes for the novel RNA molecule and, after infection, expresses it in a target cell, whereby a cytoplasmic replicon is made which is produced continuously.

In connection with this measure, it is advantageous that the DNA construct or the recombinant virus continuously produces translatable RNA molecules in the target cell, whereby it is possible to provide for a fairly long term and/or strong expression of the foreign gene. Here, the expression is controlled not only via RNA replication and translation but (especially also) via transcription. Even in the case of a weak promoter, a cytoplasmic replication which can be maintained only for a short period and an inefficient translation, it is nevertheless possible in this way to express the foreign gene efficiently. It is even possible to dispense with RNA replication in the cytoplasm.

In the case of the recombinant virus, the novel RNA molecule (e.g. CVB3 replicon) is formed under the control of a eukaryotic promoter. Here, a promoter is used which is active in many tissues or is inducible or tissue-specific, such as, for exam-

ple, the myosin light chain 2 promoter which is specific for cardiac muscle. In this connection, the vector present in the nucleus may also replicate itself, in order to achieve an even higher yield.

Furthermore, the virus genome need not necessarily originate from CVB, so that also other organ-specific viruses or polytropic viruses may be considered.

However, if the RNA molecule of the invention is intended to be transferred via the virions according to the invention, measures must be taken in order to provide the replaced coding sequences for the generation of the virions.

In view of the above, the invention further relates to a helper construct for complementing the coding sequences replaced in the RNA molecule according to the invention, with the helper construct being preferably a helper plasmid or a viral vector, which plasmid or vector codes for at least one of the replaced sequences in a translatable manner. Alternatively, the helper construct may also be a helper cell which has been transfected stably with helper DNA coding for at least one of the replaced sequences.

Accordingly, the invention also relates to a method for generating the virion according to the invention, comprising the steps:

- transfecting host cells with the vector plasmid according to the invention, and

- complementing the replaced sequences in the host cell by the helper construct according to the invention.

If the helper construct is a plasmid or a viral vector, the host cell is cotransfected with the vector plasmid and the helper construct, leading to the formation of virions in the host cell, which are formed by CVB structural proteins and package the RNA molecule according to the invention, so that the resulting virions themselves are able to infect target cells, although they cannot go through a complete infection cycle.

If the host cell is the helper cell, all that is required is to transfect the helper cell with the vector plasmid, and the host cell provides the missing sequences in trans.

Apart from the use in science, gene therapy must be regarded as a large field of application of the present invention, and, especially for end application by doctors and smaller hospitals, the novel vector system is already offered with particular foreign genes which are required for the relevant therapy. Providing tested amplificates of CVB3 ligated with the cDNA to be transferred makes it possible to guarantee good quality assurance.

In view of the above, the invention further relates to a kit with the novel vector plasmid and the novel helper construct, and to a therapeutic composition with the novel vector plasmid and/or with the novel virions and/or with the novel RNA molecule.

In this way it is possible to allow the user to administer the vector plasmid and/or the virions immediately or else to prepare appropriate, target cell-specific applications with the aid of the helper construct and/or the RNA molecules. Apart from the materials mentioned, the kit also contains those not common materials which make possible a problem-free application in a conventional manner.

On the other hand, it is also intended to allow larger hospitals to clone specific foreign genes into the novel vector system. In view of the above, the invention further relates to a method for generating the novel vector plasmid, comprising the steps:

a) providing a cDNA coding for infectious CVB, preferably CVB3,

b) cloning the cDNA into a plasmid in a transcribable manner,

c) amplifying sequence sections of the plasmid with the aid of primers leading to an amplificate which codes for the noninfectious virus genome, and

d) ligating the amplificate to a DNA sequence for the foreign gene.

The amplificates may be present in ready-to-use form in a kit, or else specific primers for generating the amplificates are provided.

A particular advantage of this method is the use of specific primers which are chosen such that they amplify not only the desired minimum portions of the future virus genome but also the portions necessary for plasmid reproduction in bacteria, for example ampicillin resistance gene, replication origin and transcription promoter.

Said amplificates then just have to be ligated with the foreign gene.

Available primers are the primers SEQ ID NO. 1 to SEQ ID NO. 4 mentioned below.

In the same way it is also possible to generate the helper construct according to the invention, the method comprising the steps:

a) providing a cDNA coding for infectious CVB, preferably CVB3,

b) cloning the cDNA into a plasmid in a transcribable manner, and

c) amplifying sequence sections of the plasmid with the aid of primers leading to an amplificate which codes for the replaced coding sequences.

The primers SEQ ID NO. 5 to SEQ ID NO. 13 mentioned below are available here for amplification.

In order to make it possible to generate a vector plasmid according to the invention with specific foreign gene and adapted minimal virus genome in routine operation in a simple manner, the invention further relates to a kit with

- a plasmid containing cloned cDNA for infectious CVB, preferably CVB3, and

- the primers required for amplification of sequence sections for generating the vector plasmid and/or the helper construct, or

- ready-to-use tested DNA amplificates.

The kit may further contain the reagents required for ligating a foreign gene.

The DNA amplificates which have at least one sequence section coding for the novel RNA molecule and can be generated by PCR, are present in the kit in ready-to-use and tested form and are easier to handle than if a PCR on the basis of the primers had to be carried out first. In this way, the problem of polymerase error rates is removed, because the end user can produce the "tailor-made" vector system more safely using tested and ready-ligatable amplificates than using amplificates prepared by himself.

Furthermore, the foreign gene in the novel RNA molecule may be used not only immediately for gene therapy purposes but also for complementing a vector which lacks parts of the genome, similar to the abovedescribed system of vector plasmid and

helper construct. This method is particularly advantageous for vector systems based on DNA viruses such as, for example, adenoviruses. If, in the case of a recombinant adenovirus, the missing parts of the genome were made available in trans in helper cells, there would be the risk of the vector recombining with the supplementing gene functions from the helper cell so that a wild-type virus would be formed, with the immediately obvious risks and disadvantages.

The invention therefore also relates to the use of the novel RNA molecule for generating recombinant viruses or virions, preferably with DNA genome, and the foreign gene coding for gene functions lacking in the DNA genome.

In this connection, it is advantageous that the recombinant CVB3 genome is always present as RNA in the cell so that recombination of the DNA genome of recombinant viruses or virions is impossible.

The RNA is translated in the cell into protein which is used for replicating or packaging the DNA genome. In this connection it is also possible to use any RNA vector system, but preferably the CVB3 vector system is used. The advantages of this method are founded on the fact that recombination of the DNA genome can be ruled out with certainty.

In view of the above, the invention also relates to a method for generating recombinant DNA viruses or DNA virions whose DNA genome lacks particular gene functions, in which method the missing gene functions are provided via a recombinant vector system with RNA genome.

Consequently, the invention relates to a universal vector system which, however, can be used advantageously for cardiac myocytes and in which translatable RNA molecules containing a foreign gene and a "defective" virus genome are transduced into a target cell in which the foreign gene is efficiently expressed in order to achieve the desired, for example therapeutic, purposes. The invention further provides methods and kits which can be used by the user to prepare the RNA molecules, vector plasmids and/or virions for the particular case of application.

It is understood that the features mentioned above and those still to be illustrated below can be used not only in the combinations indicated in each case but also in other combinations or on their own, without leaving the scope of the present invention.

Further features and advantages of the invention arise from the following description of preferred embodiments.

The examples below are illustrated on the basis of the attached drawing in which:

Fig. 1 shows schematically genomic CVB-RNA and translated polyprotein,

Fig. 2 shows schematically a CVB3 plasmid;

Fig. 3 shows an example of a recombinant RNA molecule;

Fig. 4 shows schematic examples of vector plasmids; and

Fig. 5 shows schematic examples of helper plasmids.

Example 1: CVB3 genome and CVB3 cDNA

Coxsackie viruses are representatives of the genus enterovirus in the picornavirus family. Under natural conditions, Coxsackie viruses cause diseases only in humans, but initial isolation of Coxsackie viruses is most successful in newborn mice which also serve to differentiate the viruses into two groups:

Group A with 23 serotypes and group B with 6 serotypes.

CVB, especially CVB3, are known as common pathogens of viral inflammations of the cardiac muscle, which can manifest themselves both in this acute form and also in chronic forms. Myocarditis is often lethal in infants.

Like all picornaviruses, Coxsackie viruses also have icosahedral nucleocapsids consisting of four virus proteins, VP1, VP2, VP3 and VP4. Whereas proteins VP1, VP2 and VP3 form the outer envelope, VP4 is located on the inside of the particles and is associated with the single-stranded RNA genome. The genome is infectious per se; if it is taken up by a cell under suitable conditions, even the purified RNA is able to induce an infection, because it has plus-strand orientation, i.e. the virus proteins can be translated from the RNA without an intermediate step. The 3′ end of the genomic RNA is polyadenylated and a small, virus-encoded protein V_{pg} is covalently bound to the 5′ end.

Fig. 1 shows a schematic example of the CVB3 genome. The genome contains a single open reading frame coding for a precursor protein. This polyprotein is cleaved proteolytically into the various viral components, when it is still being synthesized.

The already mentioned capsid proteins VP1-VP4 originate, as indicated, from regions 1A to 1D of the polyprotein and Vpg originates from region 3B. Regions 2A and 3C code for proteases cleaving the polyprotein. The proteins originating from regions 2B and 2C are linked to the host specificity of the viruses.

Region 3D codes for an RNA-dependent RNA polymerase which carries out replication of the RNA genome in the host cell.

At the 5′ and 3′ ends the genome also contains nontranslated regions (NTR), and the NTR region at the 5′ end has a distinct secondary structure and makes possible ribosomal binding, i.e. allows translation of the genome into the polyprotein.

The complete nucleotide sequence of a cDNA of an infectious CVB3 variant with distinct tropism toward the heart is described by Klump et al. (loc. cit.). This infectious CVB3 cDNA is available in the construct pCB3/T7 and is shown schematically in fig. 2. A promoter (Prom) which makes possible transcription of the cDNA into the RNA genome is located in front of the 5′ end.

Example 2: Recombinant RNA molecules

In the CVB3 genome from fig. 1, particular sequence regions can be replaced by foreign genes, without losing translatability of

the thus resulting recombinant RNA molecule in the target cell. The principal structure is shown in fig. 3 and consists of the NTR region at the 5′ end, an inserted foreign gene and a noninfectious residue of the virus genome.

This noninfectious residue of the virus genome comprises, for example, sequence regions 2B-3D so that the capsid proteins VP1-VP4 and protease 2A are missing.

This recombinant RNA molecule can still be translated in the host cell, due to the still present NTR region, so that the foreign gene is expressed in the target cell. For experimental purposes, the foreign gene provided for may be a reporter gene or else an effector gene which, in the framework of a gene therapy application, complements missing functions of the target cell and/or replaces, complements or inhibits defective functions.

In a minimal construct the noninfectious virus genome has to comprise regions 3B and 3D or 3CD, i.e. regions causing replication of the recombinant RNA molecule. An RNA molecule of this kind thus undergoes cytoplasmic replication and, at the same time, translation so that the foreign gene is effectively expressed in the target cell.

Since replication is carried out at the RNA level, there is no risk of the foreign gene integrating into the genome of the host cell. Since the remaining virus genome in the RNA molecule is also noninfectious, CVB3 also does not go through a complete infection cycle, i.e. the risk of additional damage to the host cell is avoided. Owing to the fact that only a minimum propor-

tion of nonstructural proteins must be encoded on the remaining virus genome, it can further be concluded that this recombinant RNA molecule is not cytotoxic for the target cell.

If, in particular applications, strong expression of the foreign gene is unwanted or not wanted over a relatively long period, it is also possible to fill the region provided for the remaining virus genome with a buffer sequence which merely serves to make the recombinant RNA molecule the same length as the original CVB3 RNA genome. An RNA molecule of this kind would still be translated but not replicated in the target cell so that cytoplasmic translation is stopped after a certain time, due to beginning degradation of the RNA molecule.

How the recombinant RNA molecule reaches the target cell, is described in example 6, but first generation of the recombinant RNA molecule with the aid of a vector plasmid whose preparation is described in the next example should be discussed.

Example 3: Preparation of vector plasmids

The recombinant RNA molecules can in principle be generated by any genetic engineering method; however, the use of vector plasmids which code for the RNA molecules and into which said RNA molecules can be transcribed, provides a large variety of application possibilities so that this route was preferred.

The infectious CVB3 cDNA from construct pCB3/T7 (see example 1) was cloned into the EcoR 1 cleavage site of basis vector pCR-Script™ from Stratagene. In order for the cDNA to be transcribed into RNA, the promoter of human cytomegalovirus from

plasmid pCMVβ from Clontech was additionally cloned into the Sal I cleavage site of pCR-Script™. This resulted in a plasmid called pCMV-CVB3.

This plasmid is the starting point for preparing the vector plasmids shown in fig. 4.

The vector plasmids are generated via polymerase chain reaction (PCR) with the aid of primers which are chosen such that they amplify not only the desired minimum portions of the future CVB3 vector but also the portions of the bacterial pCR-Script vector backbone, required for plasmid reproduction in bacteria, for example the ampicillin resistance gene, the replication origin, the promoter, etc.

Fig. 2 describes in Arabic numerals the starting points for the PCR reaction, which lead to the corresponding vector plasmids 1, 2 and 3.

A universal reverse primer from 5′ NTR is the sequence SEQ ID NO. 1:

5′-TTT GCT GTA TTC AAC TTA ACA ATG AAT TGT AAT GTT TTA ACC-3′

For the forward primer from 2C, SEQ ID NO. 2 is used:

5′-ATG GCT GAA CGC CAA AAC AAT AGC TGG C-3′

The forward primer used from 2B is SEQ ID NO. 3:

5′-GAT GCA ATG GAA CAG GGA GTG AAG GAC TAT G-3′

The forward primer used from 3′ NTR is SEQ ID NO. 4:

5′-TAG ATT AGA GAC AAT TTG AAA TAA TTT AGA TTG GC-3′

Using primers SEQ ID NO. 1 and SEQ ID NO. 2 results in a vector plasmid as shown in fig. 4 under 1, i.e. in which the sequence regions 2C-3D and the NTR regions at the 3′ and 5′ ends are present. The promoter for transcription into RNA is also present.

Analogously, primers SEQ ID NO. 1 and SEQ ID NO. 3 lead to vector plasmid No. 2 in which remain region 2B-3D and 3′ and 5′ NTR and the promoter of the virus genome.

Accordingly, SEQ ID NO. 1 together with SEQ ID NO. 4 result in plasmid No. 3 of fig. 4; here, neither structural nor nonstructural genes of the RNA genome remain, only 3′ NTR and 5′ NTR and the promoter are present in order to make transcription and translation in the target cell possible; an RNA coming from this plasmid can no longer be replicated, since the polymerase 3D is missing. In order to nevertheless obtain the original length of the virus genome for packaging into a virion, a filling sequence called "stuffer" is provided for.

The amplificates just described need then just be ligated at their ends with the foreign gene. The thus produced vector plasmid can then be transformed directly into E. coli.

In this way, the vector plasmids can be amplified and then be transcribed into RNA, resulting in the RNA molecules of example 2, after appropriate purification.

However, a preferred strategy is to cotransfect said vector plasmids together with helper constructs into host cells, in order to generate infectious virions whose genome, however, is the recombinant RNA molecule of example 2.

In order to make this cotransfection succeed, the helper constructs must complement the virus genome sequences replaced by the foreign gene.

Example 4: Preparation of helper constructs

Starting from plasmid pCMV-CVB3 of example 3, the sequence regions missing in each vector plasmids of fig. 4 are amplified using specific PCR primers. These amplificates do not contain any parts of the bacterial vector.

These amplificates may then be introduced into host cells using a viral vector; on the other hand, it is also possible to transfect a helper cell stably with said amplificates so that the helper cells serve as host cells for transfection with the vector plasmid and provide in trans the missing sequences of the virus genome.

Preference is given to helper constructs which are likewise plasmids and which are stably or transiently transfected into host cells so that they can then be transcribed into RNA which in turn is translatable, in order to generate the structural

and nonstructural proteins which are not encoded by the vector plasmid itself.

For this purpose, it is necessary to clone a promoter, for example CMV promoter, and an IRES (internal ribosomal entry site) into, for example, the pCR-Script™ plasmid. The amplificates with the helper portions of CVB3 are cloned behind said promoter and IRES. The IRES increases the translation efficiency of the helper portions, and it is possible to use, for example, the IRES of EMVC (Encephalomyocarditis Virus), the EMCV-IRES from Clontech.

Fig. 5 shows helper plasmids which can complement the vector plasmids of fig. 4.

For sequence regions 1A-1D the primers SEQ ID NO. 5:

5'-A GAC TCT AGA CAG CAA AAT GGG AGC TCA AGT ATC AAC GC-3'

and SEQ ID NO. 6 are used:

5'-A TAT GCG GCC GCC TAA AAT GCG CCC GTA TTT GTC ATT GTA GTG ATG C-3'

For region 2A primer SEQ ID NO. 7

5'-A TAT GCG GCC GCC AGC AAA ATG GGA CAA CAA TCA GGG GCA GTG TAT GTG G-3'

or SEQ ID NO. 8

5′-CTT AAG ATG GGA CAA CAA TCA GGG GCA GTG TAT-3′

is used as forward primer and SEQ ID NO. 9 is used as reverse primer:

5′-A TAT GGG CCC CTA CTG TTC CAT TGC ATC ATC TTC CAG C-3′

For sequence region 2B the primer SEQ ID NO. 10

5′-A TAT GCG GCC GCC AGC AAA ATG GGA GTG AAG GAC TAT GTG GAA CAG C-3′

and the primer SEQ ID NO. 11 are used:

5′-A TAT GGG CCC CTA TTG GCG TTC AGC CAT AGG GAT TCC G-3′

For the region 2B-3D the primer SEQ ID NO. 12

5′-A TAT GCG GCC GCC AGC AAA ATG GGA GTG AAG GAC TAT GTG GAA CAG C-3′

and the primer SEQ ID NO. 13 are used:

5′-A TAT GGG CCC CTA AAA GGA GTC CAA CCA CTT CCT GCG-3′.

In this way, helper plasmids are generated which can be amplified in bacteria and can be transcribed into RNA which in turn can be translated in order to complement the translation products of the vector plasmids such that virions can be formed, as will be described now in example 5.

Example 5: Preparation of infectious virions

While it is in principle possible to prepare the RNA molecules of example 2 in any way and then to package said RNA molecules into virus protein to give infectious virions, a more efficient way is to transfect the helper cells mentioned in example 4, which have been stably transfected with the sequences missing in the vector plasmid, with the appropriate vector plasmid which is complemented in trans by the host cell. In this way virions are formed which can be used after appropriate purification.

One way which introduces more variability is to cotransfect host cells with the vector plasmid of example 3 and the corresponding complementing helper plasmid of example 4, thus producing infectious virions which package the RNA molecules of example 2.

Example 6: Application in gene therapy

The aim of the application in gene therapy is to transduce the RNA molecules of example 2 into target cells and to cause expression of the foreign gene there. In order to enhance expression, it is advantageous if the RNA molecules are not only translated but also replicated in the target cells. A pure translation is possible using vector plasmid 3 of fig. 4, while vector plasmids 1 and 2 lead in the abovedescribed way to RNA molecules which also replicate, resulting in stronger expression.

First, it is now possible to generate the RNA molecules in host cells and then transfect said RNA molecules into the target cells, for example via lipofection.

However, a more efficient and more specific application is carried out via infecting the target cells with the virions of example 5. Owing to their capsid, said virions are in fact infectious, i.e. they can enter the target cells and release the RNA molecules packaged by them for translation and, possibly for replication. Since the RNA molecules themselves are noninfectious, no new virions are generated in the target cells. To recapitulate: the noninfectious virus genome which is part of the recombinant RNA molecules has deficits or modifications with respect to particular structural and possibly nonstructural proteins. These deficits are complemented, for example by the helper plasmids of example 4, only for preparing the virions.

If the vector system described thus far is constructed on the basis of CVB, the virions have a particular tropism toward the heart, and the sequences and therefrom translated proteins of the virus genome are particularly well adapted to said host cells. This results in foreign genes which can be transduced in this way into cardiac myocytes without problems and be expressed there.